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Isolation and Characterization of Mutants of *Arabidopsis thaliana* with Increased Resistance to Growth Inhibition by Indoleacetic Acid-Amino Acid Conjugates[1]

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Two mutants of *Arabidopsis thaliana* that are resistant to growth inhibition by indole-3-acetic acid (IAA)-phenylalanine have been isolated. Both mutants were 2- to 3-fold more resistant than wild type to inhibition by IAA-phenylalanine, IAA-alanine, and IAA-glycine in root growth assays. The mutant *icr1* (but not *icr2*) also shows some resistance to IAA-aspartate. Studies using ^{3}H-labeled IAA-phenylalanine showed that the uptake of conjugate from the medium by *icr1* was the same as wild type and was reduced by about 25% in *icr2*. No differences in hydrolysis of the exogenous conjugate were detected between the mutants and their wild-type parents. There was no significant metabolism of the IAA released from the [^{3}H]IAA-phenylalanine, whereas exogenous [^{3}H]IAA was rapidly metabolized to two unidentified products considerably more polar than IAA. Analysis of a cross between *icr1* and *icr2* indicated that these mutations were at distinct loci and that their effects were additive, and preliminary mapping data indicated that *icr1* and *icr2* were located at the top and bottom of chromosome V, respectively.

Most of the IAA in plants is found not as the free acid but in the form of conjugates. These conjugates are of two general forms, with IAA being conjugated either to sugar moieties via an ester linkage or to amino acids or peptides via an amide linkage (Cohen and Bandurski, 1982). In Arabidopsis seedlings the amounts of free, ester-conjugated, and amide-conjugated IAA are approximately 0.029, 0.18, and 17.1 $\mu g\ g^{-1}$ fresh weight, respectively (Campell and Town, 1991).

Several roles have been postulated for auxin conjugates, and feeding studies show that IAA conjugates per se are biologically inactive and demonstrate auxin activity only when hydrolyzed by plant enzymes to release the free auxin (Hangarter and Good, 1981; Bialek et al., 1983). In maize IAA ester conjugates appear to serve as a storage form in the seed, with their hydrolysis providing IAA for early growth and development prior to the onset of de novo biosynthesis (Epstein et al., 1980). Other roles that have been suggested for conjugates include protection against peroxidation and other forms of degradation, as forms for transport, possibly including tissue targeting, and as a general mechanism for hormonal homeostasis (Cohen and Bandurski, 1982). In addition, conjugation has long been recognized as a way for plants to detoxify excess hormone following exposure to the broadleaf herbicide 2,4-D as well as other auxins (Feung et al., 1974, 1975; Caboche et al., 1984). Conjugation may also play a role in the catabolism of endogenous IAA in some species. Tuominen et al. (1994) demonstrated the conversion of IAA-Asp to oxindole-3-acetyl-*N*-Asp and oxindole-3-acetic acid following the initial conjugate formation in *Populus* hybrids, suggesting that this may be a normal route for the breakdown of endogenous IAA.

Although ester-linked forms make up the predominant conjugate in maize and have been extensively studied by Cohen and Bandurski (1982), amide conjugates appear to account for the bulk of conjugated IAA in most dicots. Only a few amide-linked moieties have been found in the endogenous IAA conjugates of dicot species examined to date. IAA-Asp and IAA-Glu have been identified as the principal natural conjugates in cucumber (Sonner and Purves, 1985) and soybean (Cohen, 1982; Epstein et al., 1986), whereas in *Phaseolus vulgaris*, the majority of amide-linked IAA is found attached to a 3.6-kD peptide (Bialek and Cohen, 1986). IAA-Ala was recently identified as an endogenous IAA conjugate in *Picea abies* (Ostin et al., 1992). IAA-Gly and IAA-Val were reported in *Parthenocissus tricuspidata* crown gall (Feung et al., 1976), although their occurrence may have been due to the hormonal status of these crown gall cultures and thus may not reflect the natural conjugates in this species.

To date, all plants that have been examined appear to be able to hydrolyze both naturally occurring conjugates and a number of other amide conjugates that have not been found in any plant species (Hangarter and Good, 1981; Bialek et al., 1983). It is not known whether the synthesis or hydrolysis of the various amide conjugates by a single

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Abbreviations: Atmin, *Arabidopsis thaliana* minimal medium; Col, Columbia ecotype of Arabidopsis; IAA-Ala, indole-3-acetyl-Ala; IAA-Asp, indole-3-acetyl-aspartate; IAA-Glu, indole-3-acetyl-glutamate; IAA-Gly, indole-3-acetyl-Gly; IAA-Phe, indole-3-acetyl-Phe; IC_{50}, conjugate concentration producing 50% reduction in growth compared with the control; *icr*, IAA conjugate resistant; MS, Murashige-Skoog medium; Ws, Wassilewskija ecotype of Arabidopsis.

plant species is carried out by single enzymes with broad specificity or by multiple enzymes with restricted specificity. Progress in the molecular biology and biochemistry of enzymes involved in auxin conjugate metabolism has been relatively slow. Kuleck and Cohen (1993) reported substantial purification of an IAA-amino acid conjugate hydrolyzing activity from an embryogenic carrot culture, and the enzyme was active against IAA-Ala, IAA-Phe, and IAA-Trp but not against IAA-Asp, IBA-Ala, or the dipeptide Trp-Ala. Szerszen et al. (1994) recently cloned a cDNA from maize (*iaglu*), of which the protein product is capable of synthesizing IAA-Glc from IAA and UDP-Glc.

Until recently, there were only two reports of genetic alterations in auxin conjugate metabolism in plants. Slovin and Cohen (1988) reported the isolation of a giant mutant of *Lemna* from tissue culture regenerants. The mutant had higher levels of free IAA at several stages of the 45-d culture cycle and had no detectable IAA conjugates, ester, or amide; however, further analysis of this mutant has not been reported. More recently, we identified a cell line apparently defective in IAA conjugate accumulation from among a collection of tumors that we had induced on *Arabidopsis thaliana* by exposure to ionizing radiation (Town et al., 1994).

In this paper we describe the beginning of an investigation of IAA conjugate metabolism by the isolation and characterization of Arabidopsis mutants resistant to growth inhibition by IAA conjugates. In a similar study, Bartel and Fink (1995) also isolated IAA conjugate-resistant mutants. The mutated gene in one of their conjugate-resistant strains was cloned by chromosome walking and was shown to encode an amidohydrolase capable of hydrolyzing IAA conjugates in vitro. Knee and Hangarter (1993) have also isolated Arabidopsis mutants resistant to IAA-amino acid conjugates, but details of their work have not yet been published.

MATERIALS AND METHODS

Media and Growth Conditions

Seeds were surface-sterilized in 30% bleach, 0.1% Triton X-100 for 10 min and rinsed extensively with distilled water. Growth medium was Arabidopsis nutrient salts (Atmin; Somerville and Ogren, 1982), MS (Murashige and Skoog, 1962), and one-half-strength Gamborg's B5 (Gamborg et al., 1968). Medium was solidified with Bacto Difco agar (Difco, Detroit, MI). IAA-Phe and IAA-Ala were of the L form, whereas IAA-Asp was of the DL form. We use the abbreviation IAA-Asp to refer to the L form when describing endogenous conjugates and to refer to the DL form for the exogenous conjugate used in this study. All conjugates were obtained from Aldrich, except IAA-Gly, which was from Research Organics (Cleveland, OH). Stock solutions were made in water, filter-sterilized, and checked for purity by HPLC. Agar was allowed to cool to 50°C after autoclaving before IAA conjugates or IAA were added. Seeds were cold-treated for 24 h at 4°C in the dark and incubated at 24°C under constant light (cool-white fluorescent, approximately 40 μmol/m^2) that was filtered by yellow no. 2208 acrylic (Acrylite, Cyro, Rockaway, NJ) to minimize the photodegradation of the indolic compounds (Stasinopoulos and Hangarter, 1990). For root growth experiments seeds were plated 5 to 10 mm apart in straight lines (approximately 20 seeds for each condition) and plates were incubated with the agar surface in a vertical orientation. Root lengths were determined to the nearest millimeter for all seeds that germinated, and the average for each group was determined. Thus, average root lengths in the range 0 to 1.0 mm arise from populations in which there is no measurable root growth in some of the germinated seed.

Mutant Isolation

Two different Arabidopsis ecotypes were used in this study: Col and Ws seeds were plated (500–1000 seeds per 100-mm-diameter Petri dish) on agar containing Arabidopsis nutrient salts and 200 to 300 μM IAA-Phe. Possible resistant seedlings were identified after 3 to 4 weeks by their increased growth and vigor compared with the general population and were transferred to soil and allowed to set seeds for further analysis.

Synthesis of Radiolabeled Conjugates

Radiolabeled conjugates were synthesized by the method of Cohen (1981), modified for the low concentrations of substrate involved using ^{3}H- rather than carbon-labeled IAA. The reaction mixture containing 100 μCi of [^{3}H]IAA (Amersham, 27 Ci/mmol), 1.0 μmol of dicyclohexylcarbodiimide, and 1.5 μmol of Phe *t*-butyl ester in 1000 μL of anhydrous acetonitrile was incubated in the dark at 4°C for 48 to 72 h. When the reaction was complete, the products were taken to dryness under argon, and the ester groups were removed from the intermediate by incubation at 60°C for 3 to 4 h in 2 N KOH in 50% methanol. Conversion of [^{3}H]IAA into the ester intermediate and hydrolysis of the butyl ester to release the product were followed by HPLC. The final product was purified using a 19- × 300-mm μBondapak C_{18} column (Waters) and a Radiomatic *A*525 detector (Packard).

Uptake and Metabolism Studies

Seedlings were removed from growth plates and placed, either roots only or the entire plant, in MS (pH 5.8, no hormones) containing conjugate in microtiter wells (Falcon 3072 96-well plates, Becton Dickinson) and incubated under standard growth conditions. In experiments in which only roots were immersed, dishes were incubated in a water-saturated atmosphere. In some experiments the aerial and submerged parts of root-incubated plants were analyzed separately to follow the translocation of label from root to shoot. After the appropriate incubation period, seedlings were removed, rinsed with water, and then homogenized in methanol. The methanolic extract was either sampled directly for liquid scintillation counting or cleared by centrifugation and subjected to HPLC analysis. Samples involving conjugate hydrolysis were analyzed by reverse-phase chromatography (Microsorb-MV C_{18}, 4.6 mm × 25

cm; Rainin Instrument, Ridgfield, NJ) using an isocratic solvent system consisting of 50% methanol, 0.5% acetic acid at a flow rate of 1.0 mL min^{-1}. Detection was performed using fluorescence (excitation 280, emission 350 nm) and flow scintillation counting with a Radiomatic *A*525 detector (Packard). To analyze metabolites following [^{3}H]IAA feeding, the following gradient program was used (solvent A is methanol; solvent B is 1% acetic acid): 0 min, 30% solvent A; 8 min, 30% solvent A; 13 min, 100% solvent A; and 19 min, 100% solvent A.

Mapping Procedures

For genetic analysis, a cross was performed between CW6-6 (*icr1*; a kanamycin-sensitive, conjugate-resistant segregant from the original isolate) and MSU25 (*er, gl1, hy2, tt6*). Visible markers were scored in the F_2 progeny. Because the ranges of the root lengths of wild type and the mutant populations that were grown on the IAA-Phe overlap, the conjugate-resistance phenotype of each F_2 individual was scored by determining the average root length on 100 μM IAA-Phe of the F_3 populations from each selfed F_2 individual. Plants for DNA isolation were grown from F_3 seed populations in liquid MS under constant illumination and shaken at approximately 50 rpm. DNA was isolated by the cetyltrimethylammonium bromide procedure essentially as described by Keller (1992). For restriction fragment length polymorphism analysis, Southern blotting was done by standard procedures, probes were labeled by random priming (Feinberg and Vogelstein, 1984), hybridization was in Church's buffer at 63°C (Church and Gilbert, 1984), and blots were washed twice for 30 min each time in 0.2× SSC, 0.1% SDS at 60°C. Cleaved amplified polymorphic sequence (Konieczny and Ausubel, 1993) and simple sequence length polymorphism (Bell and Ecker, 1994) markers were analyzed as described by their originators. Recombination values were calculated using Linkage-1 (Suiter et al., 1983) and were converted to map distances (Kosambi, 1944).

RESULTS

Mutant Isolation

IAA conjugates inhibit the growth of Arabidopsis seedlings, probably because the conjugates are hydrolyzed and the IAA released is phytotoxic. To identify the mutants that were defective in conjugate hydrolysis, mutagenized seed populations were plated on Atmin containing 200 to 300 μM IAA-Phe. IAA-Phe was chosen because it demonstrated good phytotoxicity, and Atmin medium was preferred to MS because on the latter medium the seedlings under selection began to form callus, presumably due to the released IAA. We screened both ethyl methanesulfonate-mutagenized Col seeds (Lehle Seeds, Tucson, AZ) and the original Feldmann T-DNA collection in the Ws ecotype (Feldmann and Marks, 1987; obtained from the Arabidopsis Biological Resource Center, Ohio State University, Columbus). After retesting possible mutants, one ethyl methanesulfonate- and one T-DNA-derived line (CW204 and CW6, respectively) were retained for further analysis. Segregation analysis of the T-DNA-derived mutant after self-fertilization indicated that the mutation causing conjugate resistance was not associated with either kanamycin resistance or the production of nopaline. A kanamycin-sensitive, conjugate-resistant segregant of CW6, designated CW6-6, was used for the experiments described below. Since the mutants showed resistance to several different IAA-amino acid conjugates, the genetic loci conferring conjugate resistance were named *icr* for IAA conjugate resistance rather than for the specific conjugate on which they were isolated. Subsequent analysis (see below) showed that the mutations in CW6-6 and CW204 were not allelic, and they were designated *icr1* and *icr2*, respectively.

Quantitative Analysis of Wild-Type and Mutant Responses to IAA Conjugates

Using the root growth inhibition assay, we have examined the sensitivity of wild-type and mutant plants to IAA-Phe, IAA-Ala, IAA-Asp, and IAA-Gly. Representative data for the growth of one mutant (CW6-6) and its parental ecotype (Ws) on different concentrations of IAA-Phe are shown in Figure 1. In the absence of IAA-Phe the phenotypes of wild-type Ws and mutant CW6-6 were very similar, although root growth was slightly faster in the mutant. Analysis of all of the root growth data at 10 d for each mutant and its respective parent showed that both mutants

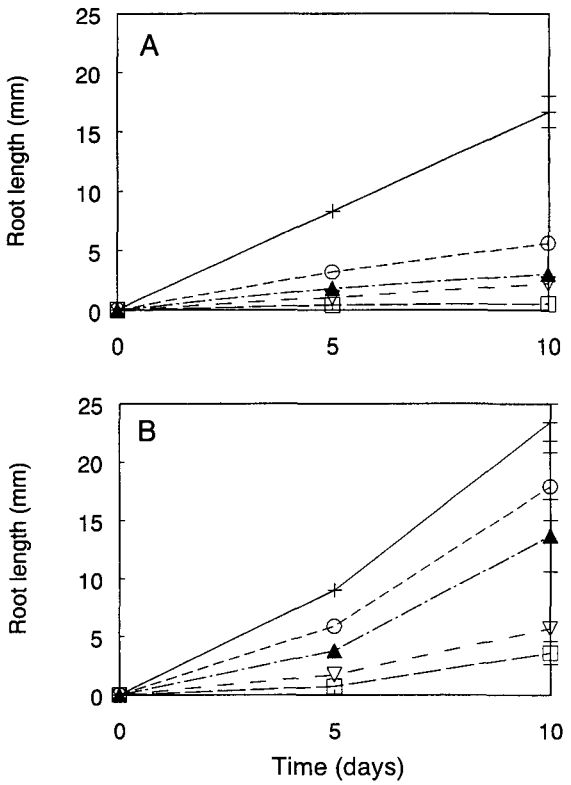

Figure 1. Root growth of wild-type (A, Ws) and mutant (B, CW6-6, *icr1*) seedlings on Atmin agar containing different concentrations of IAA-Phe, as indicated by the following symbols: +, control; ○, 25 μM; ▲, 50 μM; ▽, 100 μM; □, 150 μM.

had slightly increased rates of root growth (average lengths at 10 d: Ws, 19.8 ± 1.9 mm; CW6-6, 27.7 ± 3.1 mm; Col, 20.0 ± 2.4 mm; CW204, 30.6 ± 2.7 mm). These differences are not dramatic but are significant at the 5 and 1% levels, respectively. Incubation of seedlings on a range of increasing concentrations of IAA-Phe resulted in a progressive decrease in the amount of root growth, with the inhibition being much more marked in wild-type than in mutant seedlings.

To compare responses both within and between experiments, root length after each treatment is expressed as a percentage of the growth of untreated controls in the same experiment. The responses of the two mutants and their respective wild-type parents to increasing concentrations of IAA-Phe from a number of replicate experiments are shown in Figure 2. Root inhibition assays were performed on various growth media to determine whether the different media affected the mutants' response to the conjugate. From each independent experiment the IC_{50} was deduced by interpolation between data points above and below the 50% inhibition level. The results from both the mutants and their parental wild types for a number of IAA-amino acid conjugates analyzed on different growth media are summarized in Table I. Several points can be noted.

In general, the two wild types (Ws and Col) showed similar responses to each other, both on different growth media and to different conjugates. IAA-Phe, IAA-Ala, and IAA-Gly were approximately equally effective as growth inhibitors, whereas IAA-Asp (DL form) was between 10 and 60 times less effective on a molar basis, depending on the strain and media conditions used. The resistance of each mutant relative to its wild-type parent can be estimated from the ratio of the mutant:wild-type IC_{50} values for each set of conditions (Table II). The degree of resistance varied somewhat according to the conjugate and medium that were used for the assay. However, on average, CW6-6 (*icr1*) was approximately 2.3, 3.0, 3.2, and 1.7 times more resistant to IAA-Phe, IAA-Ala, IAA-Gly, and IAA-Asp, respectively, than its wild-type parent, and CW204 (*icr2*) was

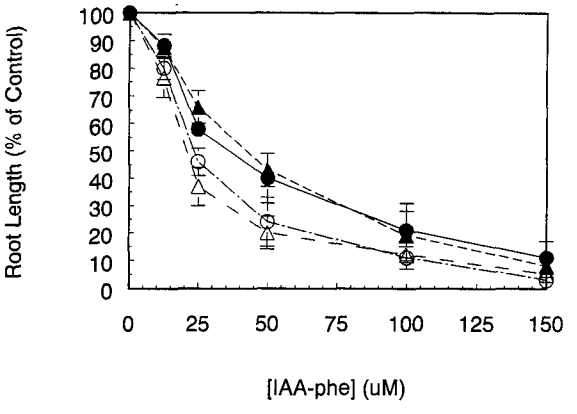

Figure 2. Inhibition of root elongation in wild-type and mutant strains in the presence of varying concentrations of IAA-Phe. Measurements were made after 5 d of incubation and are expressed as a percentage of the growth observed on control plates lacking IAA-Phe. Data are means ± SE from three to four independent determinations. Ws, △; CW6-6 (*icr1*), ▲; Col, ○; CW204 (*icr2*), ●.

approximately 2.2, 2.4, 2.3, and 1.2 more resistant than its Col parent. Although these values are crude averages, they indicate that not only was IAA-Asp (DL form) a poor growth inhibitor, but also that the mutations in strains CW6-6 and CW204 (especially the latter) had a much smaller effect on resistance to this conjugate than to the other three. Our root length data showed less variability on Atmin and B5 than on MS, as determined by the coefficient of variation (the ratio of SE to mean) that was obtained on the three media. Therefore, Atmin was routinely used for subsequent genetic analysis of the mutants.

Conjugate resistance could be due to resistance to the conjugate per se or to resistance to the products of conjugate hydrolysis (IAA and the cognate amino acid). Therefore, we examined the resistance of the two mutants and their wild-type parents both to free IAA and to free amino acids, using the same root growth inhibition assay. Figure 3 shows that there was no difference in the sensitivity of either of the mutants and their wild-type progenitors to free IAA. Since seedlings were completely inhibited by micromolar concentrations of IAA, it is unlikely that they were ever exposed to substantially higher concentrations of the released amino acids. Our tests indicated that none of the amino acids tested (L-Phe, L-Ala, Gly, and DL-Asp) had any effect on root growth up to levels of 150 μM, the highest concentration of conjugate used in any of our experiments (data not shown).

Analysis of Conjugate Uptake

To investigate the basis of IAA-conjugate resistance in the mutants, we first examined the uptake of IAA-Phe from the medium by wild-type and mutant Arabidopsis. The mutant selections were performed on Atmin medium. However, plants grown on Atmin were much smaller than those grown on MS and B5 (average weights of 15-d-old seedlings were 5, 20, and 30 mg/plant, respectively). Since the conjugate-resistance phenotype is expressed to a similar degree in plants grown on other media (Table I) and the plants grown on MS or B5 medium gave higher and more reproducible uptake than those on Atmin, B5- or MS-grown plants were used for most uptake and hydrolysis experiments. In addition, the more compact, branched root structure of the B5-grown plants made them easier to manipulate in the microtiter wells.

Fifteen-day-old plants were removed from agar and incubated in microtiter wells, either roots only or fully immersed, in medium containing [^{3}H]IAA-Phe (10^5 dpm of [^{3}H]IAA-Phe alone or with 100 μM nonradioactive IAA-Phe). At intervals the plants were removed from the wells, washed with water, and extracted in 100% methanol. The radioactivity in each extract was determined by liquid scintillation counting. The results presented in Figure 4 are for fully submerged plants, because these data showed less variability than those in which only the roots were immersed. The incubation of plants in medium containing IAA-Phe led to a rapid uptake of conjugate, which continued at a decreasing rate for at least 24 h. When Atmin-grown plants were used, both the kinetics of uptake and its magnitude (expressed per gram fresh weight) were similar

Table I. *IC_{50} values for inhibition of root elongation in wild-type and mutant Arabidopsis on different IAA-amino acid conjugates*

IC_{50} values were determined from growth data on d 5 as the concentration of IAA conjugate required to reduce root growth to 50% of control. Data are means ± SE of three to six independent determinations. Average root lengths of control plants after 5 d of growth were 6 to 9 mm on Atmin, 20 to 22 mm on B5, and 6 to 10 mm on MS.

Strain	Atmin				B5			MS		
	IAA-Phe	IAA-Ala	IAA-Gly	IAA-Asp[a]	IAA-Phe	IAA-Ala	IAA-Asp[a]	IAA-Phe	IAA-Ala	IAA-Asp[a]
			μM			μM			μM	
CW6-6 (*icrl*)	51 ± 5	70 ± 5	66 ± 24	713 ± 78	27 ± 6	37 ± 6	637 ± 44	48 ± 26	67 ± 39	768 ± 180
WS (Wild type)	23 ± 2	21 ± 1	20 ± 2	318 ± 70	14 ± 2	19 ± 1	530 ± 62	18 ± 8	18 ± 11	483 ± 85
CW204 (*icr2*)	60 ± 14	78 ± 11	72 ± 30	547 ± 130	21 ± 2	27 ± 2	680 ± 96	46 ± 25	58 ± 25	677 ± 120
Col	23 ± 1	25 ± 3	31 ± 11	403 ± 50	12 ± 2	19 ± 1	580 ± 82	20 ± 7	22 ± 9	633 ± 155

[a] IAA-Asp was the DL form; all other conjugates were the L form.

to the results for B5 plants (data not shown). Under the growth and uptake conditions used for Figure 4, there was no difference in uptake between Ws and CW6-6, either with 10^5 dpm of [^{3}H]IAA-Phe alone (data not shown) or with 10^5 dpm of [^{3}H]IAA-Phe in the presence of 100 μM nonradioactive IAA-Phe (Fig. 4A). When Col and CW204 were compared, there was a consistent reduction in uptake in CW204 compared with its wild-type parent at both low (tracer; data not shown) and high (plus 100 μM IAA-Phe) conjugate concentrations (Fig. 4B). Very similar amounts of radioactivity were taken up from medium containing 10^5 dpm of [^{3}H]IAA-Phe in the absence or presence of 100 μM carrier IAA-Phe.

When only the roots were submerged (a situation more similar to the selection conditions), uptake was reduced by about 50% compared with the fully submerged plants and was more variable. Under these conditions no difference was detected among any of the four strains. By feeding plants through the roots and then examining the kinetics of appearance of the radioactivity in roots and shoots, we observed the translocation of radioactivity from roots to shoots. We found no difference between the mutants and their wild-type parents in the rate of translocation of conjugate from roots to shoot. We also examined uptake in azide-treated and glutaraldehyde-treated plants in an effort to determine whether uptake required active metabolism. We found that uptake through the roots was reduced by about 50% by treatment of plants with 1 mM azide, as well as by overnight fixation in 2.5% glutaraldehyde, suggesting that there are both energy-dependent and energy-independent components to this uptake process. Conjugate hydrolysis under these conditions was not determined.

Hydrolysis of Assimilated IAA-Phe

Plants were incubated with 10^5 dpm of [^{3}H]IAA-Phe in the absence or presence of 100 μM carrier IAA-Phe and extracted as for the uptake experiments. One hundred microliters of the 200-μL extracts were analyzed by HPLC to determine the distribution of radioactivity among IAA-Phe, free IAA, and other metabolites. Representative chromatograms for extracts from wild-type and mutant plants after 1, 2, 4, and 8 h of incubation with 10^5 dpm of [^{3}H]IAA-Phe under fully submerged conditions (WS and CW6-6) are shown in Figure 5. By 1 h radioactivity with the retention time of IAA (4.75–5.0 min) was detectable. With longer incubation times both the proportion of radioactivity present in the plants as IAA and its absolute amount increased (see also Fig. 4). Qualitatively similar results were obtained when plants were incubated with the same amount of [^{3}H]IAA-Phe but in the presence of 100 μM IAA-Phe. In this case, however, radioactivity with the retention time of IAA appeared more slowly, presumably due to the reduced specific activity of the IAA-Phe in these incubations (data not shown).

The results from many such chromatograms for plants incubated from 1- to 48 h both in the absence and in the presence of 100 μM IAA-Phe are presented in Figure 6. To adjust for differences in uptake between individual plants, the amount of conjugate hydrolysis is expressed as the

Table II. *Relative resistance of mutants CW6-6 and CW204 on different growth media*

Ratio of mutant to wild-type IC_{50} values for the different conjugates when assayed on different growth media.

Comparison	Medium	IAA-Phe	IAA-Ala	IAA-Gly	IAA-Asp
CW6-6: Ws	Atmin	2.2	3.3	3.2	2.2
	B5	1.9	1.9	n.d.[a]	1.2
	MS	2.7	3.7	n.d.	1.6
Average		2.3 ± 0.2	3.0 ± 0.5	3.2	1.7 ± 0.5
CW204: Col	Atmin	2.6	3.1	2.3	1.4
	B5	1.75	1.4	n.d.	1.2
	MS	2.3	2.6	n.d.	1.1
Average		2.2 ± 0.2	2.4 ± 0.5	2.3	1.2 ± 0.1

[a] n.d., Not determined.

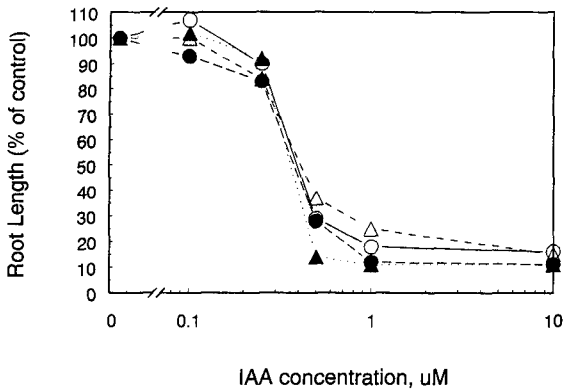

Figure 3. Inhibition of root elongation in wild type and mutants in the presence of varying concentrations of IAA. Measurements were made after 5 d of incubation and are expressed as a percentage of the growth observed on control plates lacking IAA. Ws, △; CW6-6 (*icr1*), ▲; Col, ○; CW204 (*icr2*), ●.

fraction of the radioactivity found in the plant in the form of IAA. Figure 6A shows results for plants incubated with 10^5 dpm of [^{3}H]IAA-Phe only. Under these conditions the amount of radioactivity released and detectable as IAA increased for the first 8 h. At later times, the amount of radioactivity present as IAA showed some variability and was lower in Col and CW204 than in Ws and CW6-6, suggesting that conversion of IAA to other metabolites was more rapid in Col than in Ws ecotypes. When 100 μM IAA-Phe was present during the incubation, radioactive IAA was released more slowly (Fig. 6B), but the chromatograms showed less variability and radioactivity with the retention time of IAA that was detectable throughout the 48-h incubation period. In either situation there was no apparent difference in the release of [^{3}H]IAA from [^{3}H]IAA-Phe among any of the four strains examined or for plants that had been pregrown on different media.

In these experiments no major metabolites could be identified for the IAA released from the IAA-Phe. By contrast, when plants were incubated with [^{3}H]IAA alone under similar conditions, two major metabolites with retention times much shorter than IAA were observed (Fig. 7; note that a different solvent program was used for these analyses). The noisy chromatograms at early times are presumably due to a combination of the small number of counts taken up coupled with the diversity of metabolites produced. The identity of the two major metabolites has not yet been determined. Only a small amount of radioactivity with the retention time of IAA-Asp (7.2–7.4 min) was detected. Thus, it appears that under these conditions the metabolism of exogenous IAA is different from that of the IAA released by IAA-Phe hydrolysis.

Genetic Analysis and Mapping of *icr1*

Dominance

Mutant *icr1* was originally isolated from the Feldmann collection of T-DNA-mutagenized seeds. The original isolate was heterozygous for kanamycin resistance. We examined kanamycin-sensitive segregants of the initial isolate for IAA-Phe resistance and found several kanamycin-sensitive, conjugate-resistant individuals, showing that the *icr1* mutation was not linked to the kanamycin-resistance phenotype. One of the kanamycin-sensitive, conjugate-resistant lines (designated CW6-6) was used for all of the physiological and genetic experiments reported. Previous studies have shown that some tagged mutants do not contain the entire T-DNA (Castle et al., 1993). Therefore, we also examined the conjugate-resistant lines for nopaline production and for the presence of the left and right T-DNA borders by Southern blotting. We found no evidence either for nopaline production or for the presence of T-DNA sequences in the conjugate-resistant, kanamycin-sensitive line.

For genetic analysis, a cross was performed between CW6-6 (*icr1*) and MSU25 (*er, gl1, hy2, tt6*). Analysis of root growth on IAA-Phe indicated that the phenotype of the F_1 seed was intermediate between the sensitive and resistant parents. Visible markers were scored in the F_2 progeny. Because of the variability of root growth on IAA-Phe, even within genetically homogeneous populations of each parent, it was not possible to determine the conjugate-

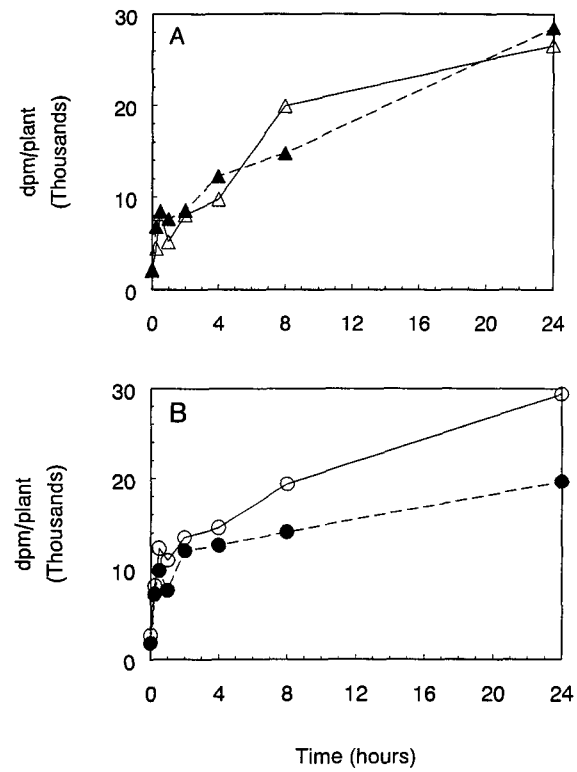

Figure 4. Uptake of IAA-Phe by wild-type and mutant plants. Fifteen-day-old seedlings grown on B5 medium were incubated fully submerged in 200 μL of MS containing approximately 100,000 dpm of [^{3}H]IAA-Phe plus 100 μM nonradioactive IAA-Phe. At intervals plants were homogenized in 100% methanol, and the activity taken up was determined by liquid scintillation counting. Results are the averages of two independent experiments with two plants per time point for each determination. Average weights of plants used in these experiments were: Ws, 31.3 ± 3.2 mg; CW6-6, 30.9 ± 4.0 mg; Col, 24.2 ± 1.5 mg; CW204, 26.5 ± 3.7 mg. A, Ws, △; CW6-6 (*icr1*), ▲. B, Col, ○; CW204 (*icr2*), ●.

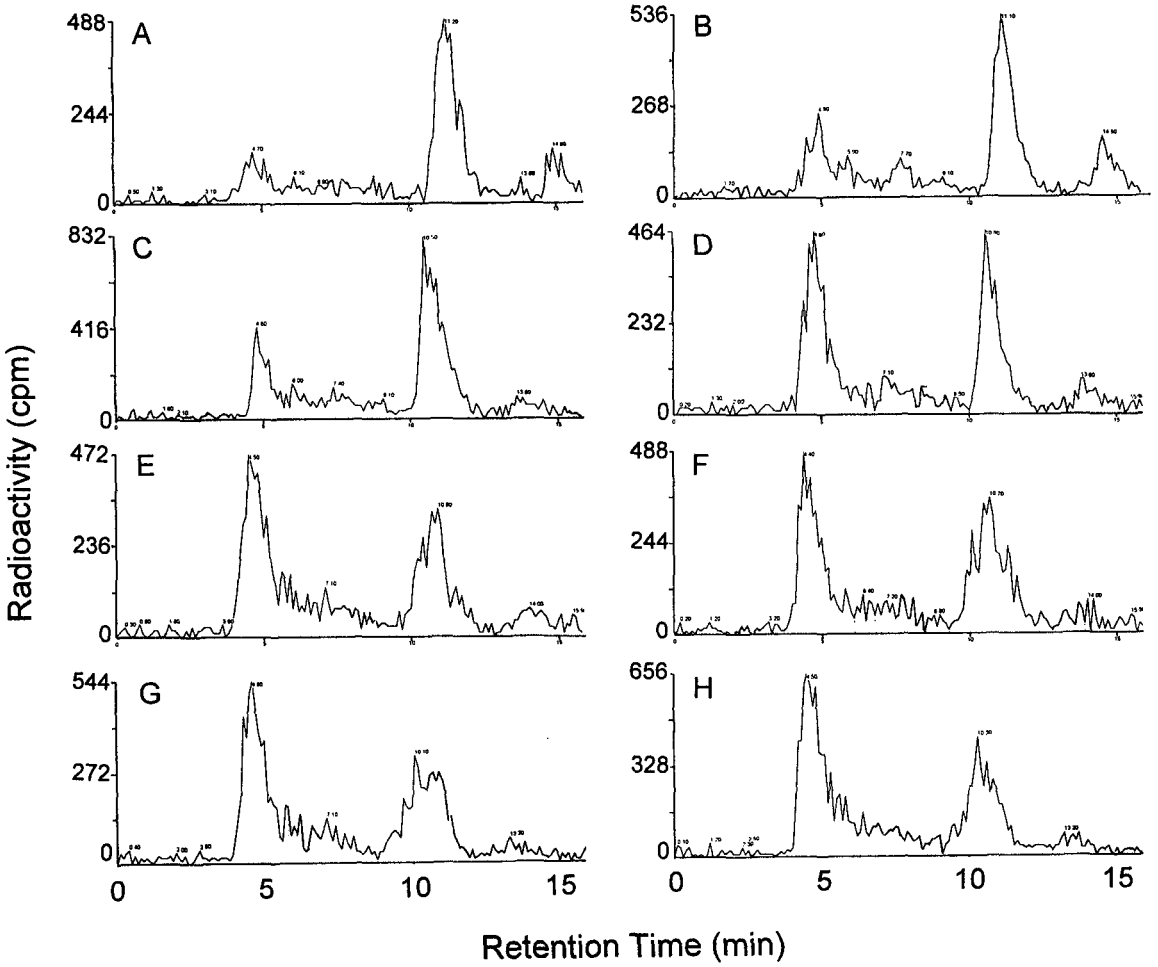

Figure 5. Representative chromatograms of IAA-Phe hydrolysis by intact plants. Fifteen-day-old seedlings were incubated for various periods in MS containing approximately 100,000 dpm of [^{3}H]IAA-Phe. At intervals plants were homogenized in 100% methanol, and the extracts were analyzed by reverse-phase HPLC using 50% methanol:50% acetic acid (1%, v/v) under isocratic conditions. Under these conditions, IAA and IAA-Phe had retention times of 4.5 to 5.0 and 9.75 to 10.25 min, respectively. Radioactivity is shown on the ordinate and retention time is shown on the abscissa. A, C, E, and G, Ws; B, D, F, H, CW6-6 (*icr1*). A and B, 1 h; C and D, 2 h; E and F, 4 h; G and H, 8 h.

resistance phenotype of F_2 progeny based upon root growth on IAA-Phe of individual F_2 segregants. Instead, conjugate resistance was scored based on the average root length on 100 μM IAA-Phe of F_3 populations derived from selfing of each F_2 individual. Segregation data for conjugate resistance in the F_2 population are shown in Figure 8. Under these assay conditions the sensitive and resistant parents (MSU25 and *icr1*) had average root lengths after 5 d of growth of 0.1 and 5.6 mm, respectively.

The segregating F_2 population did not show a bimodal distribution of resistant and sensitive individuals but rather showed a continuous distribution of average root lengths from 0 to >5 mm. To evaluate the segregation pattern, we determined the number of conjugate-sensitive individuals in the F_2 population. The response of wild-type (conjugate-sensitive) individuals appeared to be influenced by genetic background. Thus, the average root length of sensitive seedlings in the Landsberg *erecta* (Ler) background (MSU25) was 0.1 mm, whereas in the Ws background it was 0.5 mm. When the segregants with an average root length of 0.1 mm or less were treated as homozygous sensitive, the F_2 population contained 17 sensitive and 57 nonsensitive individuals, consistent with a 1:3 ratio ($\chi^2 = 0.19$; $0.9 > P > 0.5$). If a 0.5-mm cutoff was used, there were 26 sensitive and 48 resistant individuals, which is consistent with a 1:3 ratio ($\chi^2 = 4.05$; $0.05 < 0.25$). In fact, the genetic background of this F_2 population is an *erecta*/Ws mixture, so the proportion of sensitive individuals based on the Ws cutoff (0.5 mm) is a minimum estimate. Thus, it appears that the F_2 population was segregating 1:3 sensitive:resistant, which is consistent with the conjugate-resistance phenotype being controlled by a single, dominant nuclear gene. However, the individuals in the resistant class showed a continuous variation in average root lengths from that of the homozygous resistant mutant right down to those of the sensitive wild types. The presence in the F_2 population of many individuals with

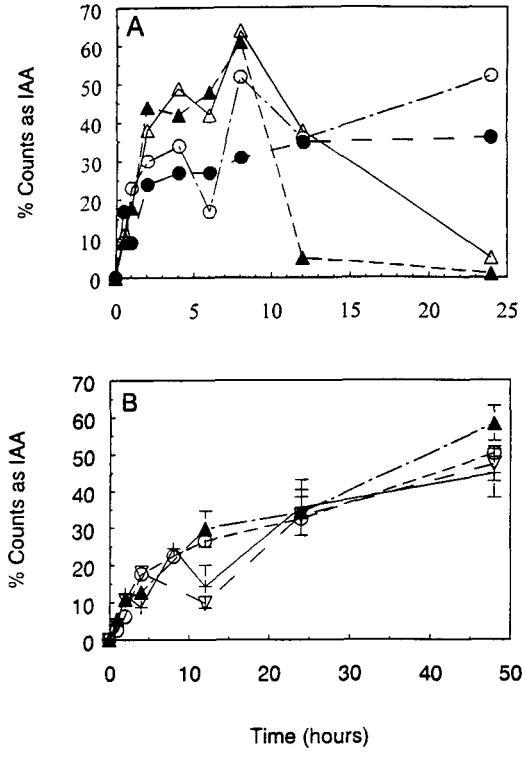

Figure 6. Release of IAA from IAA-Phe in vivo. After chromatographic analysis of the radioactivity taken up by plants as described in Figure 5, the amount of radioactivity extracted from each group of plants found as IAA is expressed as a fraction of the total activity on the chromatogram. A, Plants fed 100,000 dpm of [^{3}H]IAA-Phe only; B, plants fed 100,000 dpm of [^{3}H]IAA-Phe plus 100 μM nonradioactive IAA-Phe. Results in A are means of duplicate determinations and in B data points without error bars are from single groups of plants; others are means ± SE from three to seven determinations. Ws, △; CW6-6 (*icr1*), ▲; Col, ○; CW204 (*icr2*), ●.

conjugate-resistance phenotypes intermediate between those of the two parents suggests that the *icr1* mutation is incompletely dominant. Variations in the expressivity of the conjugate-resistance trait in the mixed Ws/Ler genetic background of the segregating F_2 population may also contribute to this wide range of resistance phenotypes. The broad distribution of conjugate-resistance phenotypes in the F_2 generation, indicative of incomplete dominance, was not due to the mixed Ws/*er* genetic background. A similar range of phenotypes was observed in a smaller population of F_2 individuals that were derived from a backcross between CW6-6 and Ws (data not shown). The analysis of the F_2 segregants of a cross between CW204 (*icr2*) and CS 3085 (*er, ap3-1*) indicates that *icr2* also exhibits incomplete dominance (W. Dong and C.D. Town, unpublished results).

Relationship between icr1 *and* icr2

To determine whether *icr1* and *icr2* were at the same genetic locus, a cross was performed between the two mutants, and the conjugate-resistance phenotype of the progeny was examined. The conjugate-resistance phenotypes of 25 F_2 progeny (expressed as average root length of derived F_3 populations after 5 d of growth on 100 μM IAA-Phe) are shown in Figure 9. In this population of 25, we found three individuals that were as conjugate-sensitive as either of the wild-type parents. Although this population size is too small to make an accurate determination of the recombination frequency between *icr1* and *icr2*, the occurrence of conjugate-sensitive segregants from this cross indicates that these two mutations are not tightly linked and reside at different genetic locations. In the same population we also observed several individuals that were significantly more resistant than either homozygous single mutant parent. The greater resistance of these presumptive homozygous double mutants suggests that the conjugate resistance conferred by the two mutations is additive. Although these crosses were performed with plants that descended from the primary mutants lines CW6-6 (*icr1*) and CW204 (*icr2*), it is unlikely that other mutations in these backgrounds might affect this outcome. Each mutation has been shown to segregate as a single nuclear gene, making it unlikely that any other secondary mutations in these lines have a discernible effect on conjugate response.

Map Locations of icr1 *and* icr2

Analysis of the segregation of *icr1* with restriction fragment length polymorphism, simple sequence length polymorphism, and cleaved amplified polymorphic sequence markers in a cross with an *erecta* ecotype (*icr1* × MSU25) indicated that *icr1* maps to the top of chromosome 5. A similar analysis of a cross between CW204 and CS3085 (*ap3*) showed that *icr2* mapped to the bottom of chromosome 5 about 10 centimorgans from *ILL1/2*, the location of sequence homologs of the *ILR* gene described by Bartel and Fink (1995) (W. Dong and C.D. Town, unpublished data), thus confirming the results of the *icr1* × *icr2* analysis. However, the weak phenotypes of both the *icr1*/+ and *icr2*/+ heterozygotes has made an accurate assignment of the conjugate-resistance phenotype of some of the segregants difficult, so these map locations are approximate. We are currently investigating ways to enhance the phenotypic difference between wild type and mutants to refine these map positions.

DISCUSSION

To study IAA conjugate metabolism in Arabidopsis, we have isolated and begun to characterize mutants of Arabidopsis resistant to growth inhibition by IAA-Phe. Although previous studies have demonstrated the presence of significant levels of both ester and amide conjugates in both Arabidopsis seedlings and plants (Campell and Town, 1991; Normanly et al., 1993), to our knowledge, the identity of these endogenous conjugates has not yet been determined. IAA-Phe was chosen as a selective agent in these studies because it was an effective growth inhibitor, and we reasoned that the hydrophobic nature of the conjugated amino acid would facilitate uptake even in the absence of a specific transport system. Both of the mutants that we

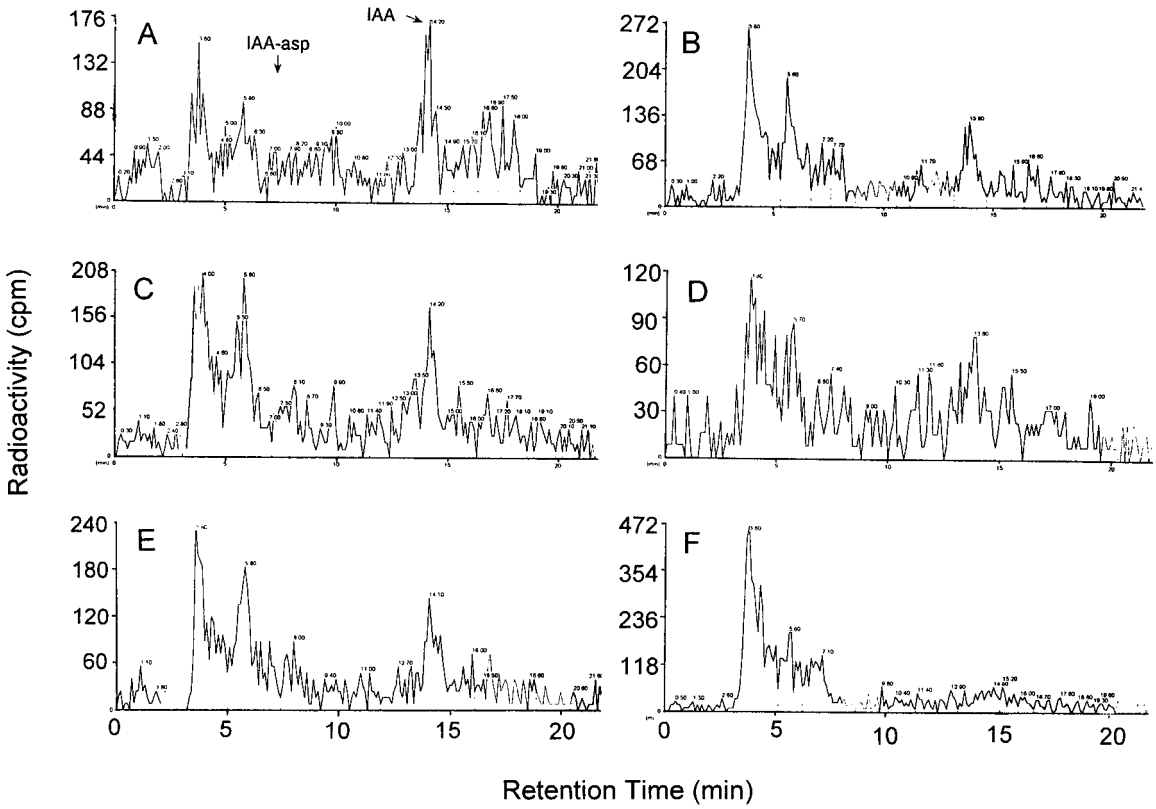

Figure 7. Metabolism of exogenous [^{3}H]IAA by Arabidopsis leaves. Leaves from 15-d-old plants grown on B5 medium were incubated for various periods in MS containing approximately 40,000 dpm of [^{3}H]IAA. At each time leaves were washed and extracted with methanol. The extracts were analyzed by HPLC with radioactivity detection and the following solvent program (solvent A is methanol; solvent B is 1% acetic acid): 0 min, 30% solvent A; 8 min, 30% solvent A; 13 min, 100% solvent A; and 19 min, 100% solvent A. Retention times of IAA-Asp and IAA under these conditions (7.2–7.4 and 13.3–13.5 min, respectively) are shown. Similar results were obtained with whole plants. A, C, and E, Ws; B, D, and F, CW6-6. A and B, 1 h; C and D, 2 h; E and F, 4 h.

selected were 2- to 3-fold more resistant than wild type to IAA-Phe, IAA-Ala, and IAA-Gly.

IAA-Asp was relatively ineffective as a growth inhibitor in Arabidopsis. CW6-6 (*icr1*) was slightly more resistant than wild-type Ws to IAA-Asp, but there was very little difference between the responses of CW204 (*icr2*) and its Col parent to IAA-Asp. Part of the reduced effectiveness of the IAA-Asp was probably due to our use of the DL form of IAA-Asp for these studies rather than the naturally occurring L form, although it is unlikely that this was the only reason for the difference in potency between this and the other three conjugates used in this study. Both mutants are as sensitive to free IAA as the wild type, indicating that these are mutants in the response to IAA conjugate and not to free IAA. In the absence of conjugate the mutants have no major phenotype, although the average root lengths of 10-d-old *icr1* and *icr2* populations (27.7 ± 3.1 and 30.6 ± 2.7 mm, respectively) are greater than those of their wild-type parents (19.8 ± 1.9 and 20.0 ± 2.4 mm, respectively).

We examined conjugate uptake both in fully submerged plants and in plants with only their roots immersed. These studies were performed on 15-d-old plants because of the small size of younger plants. Conjugate resistance based on root length was usually determined after 5 or 10 d of growth, but the difference between mutant and wild type was equally evident at 15 d, the time at which our uptake and metabolism studies were performed. Under these latter conditions, uptake was approximately 40% inhibited both by 1 mM azide and in glutaraldehyde-treated plants, suggesting the presence of both energy-dependent and energy-independent components. The amount of radioactive IAA-Phe taken up was the same in the absence and presence of the 100 μM cold competitor IAA-Phe. Although we found no alteration in conjugate uptake for CW6-6 (*icr1*), in the case of CW204 (*icr2*) under fully submerged conditions, uptake was reproducibly about 25% less than wild type. If conjugate uptake by seedlings is similar to IAA uptake and contains both saturable (carrier-mediated) and nonsaturable components, this 25% reduction in overall uptake may reflect a more severe loss of the saturable component and thus might account for this mutant's conjugate resistance.

Alternatively, it is possible that the observed reduction in average conjugate uptake is the result of a much larger and biologically significant difference in uptake in some critical part of the plant. By incubating only the roots in medium containing [^{3}H]IAA-Phe and subsequently analyzing radioactivity in roots and shoots separately, we

found that radioactivity is translocated to the aerial parts of the plant within 15 min, and by 1 h 20 to 40% of the radioactivity taken up has moved from the root to shoot. Previous studies have shown that an ester conjugate of IAA (IAA-inositol) is transported from seed to shoot in the transpiration stream in germinating maize (Chisnell and Bandurski, 1988). The mechanism by which the amide conjugate IAA-Phe was translocated from roots to shoots in our Arabidopsis experiments remains to be determined.

Uptake of [^{3}H]IAA-Phe by the plant was accompanied by its conversion to IAA and other metabolites. By 48 h most of the IAA-Phe taken up by the plants had been hydrolyzed. About 50% of the radioactivity in the plant at this time was found as IAA, and only 10 to 20% was found as unhydrolyzed IAA-Phe. No other abundant metabolites were observed. The fate of the IAA that was released from the conjugate is apparently different from that of exogenous IAA. We found that exogenous IAA was rapidly metabolized primarily to two unidentified products with HPLC retention times significantly shorter than that of IAA-Asp. One explanation for the different metabolic fates of exogenous IAA and that released from IAA-Phe could be that the IAA-Phe hydrolysis occurs in a different tissue, cell type, or subcellular compartment than that in which exogenous IAA is metabolized.

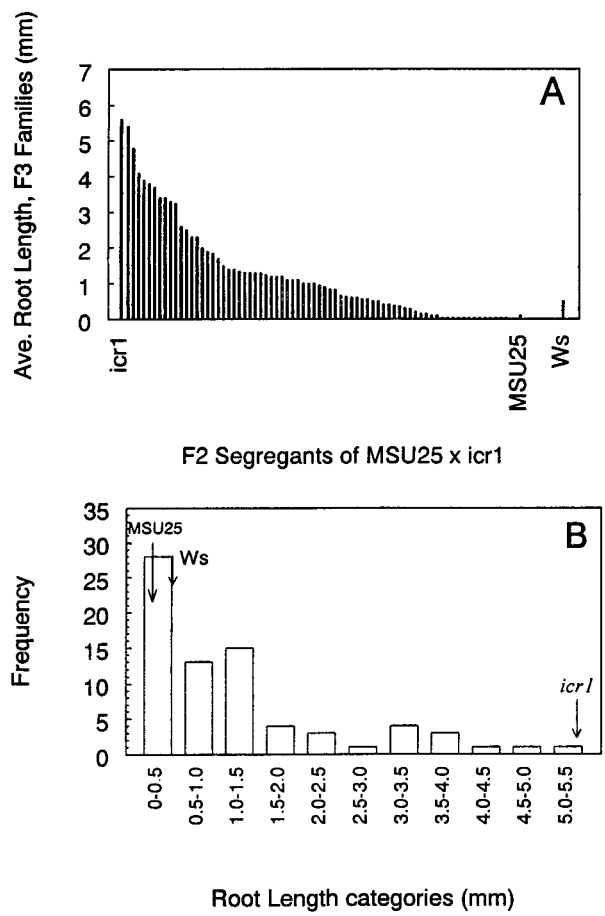

Figure 8. Analysis of conjugate resistance in the MSU25 × CW6-6 (*icr1*) segregants. A, Average root lengths after 5 d of growth on Atmin agar containing 100 μM IAA-Phe of 74 F_3 families derived from selfed F_2 segregants of a cross between CW6-6 (*icr1*) and MSU 25. B, Frequency of different root size classes within the segregating population. Arrows show the size class in which the wild types MSU25 and Ws (parent of *icr1*) and mutant CW6-6 (*icr1*) fall. Each size class represents 0.5-mm increments in average root length.

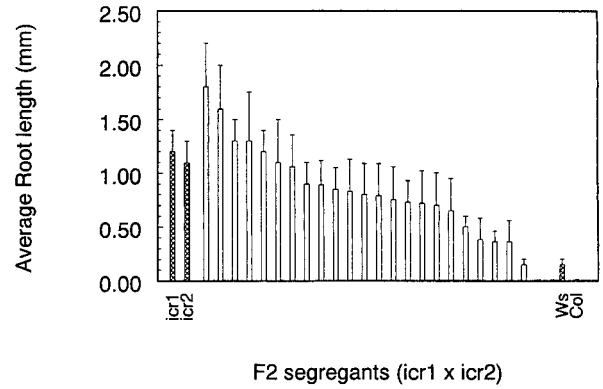

Figure 9. Average root lengths in 25 F_3 seed families from a cross of CW6-6 (*icr1*) × CW204 (*icr2*) after 5 d of growth on Atmin agar containing 100 μM IAA-Phe.

On the basis of the uptake data, it appears that the resistance of CW6-6 is not due to altered uptake, whereas the situation for CW204 is equivocal. The measurement of IAA-Phe hydrolysis did not reveal any deficiency in conjugate hydrolysis in either of the mutants. However, Bartel and Fink (1995) have recently reported the isolation from Arabidopsis of a gene (*ILR1*) that is capable of hydrolyzing various IAA conjugates, including IAA-Phe, as well as the existence of two sequence homologs of this gene (*ILL1* and *ILL2*). Based on this evidence alone, Arabidopsis apparently contains multiple genes, in which the products are capable of hydrolyzing IAA-Phe and other conjugates. The loci of both *icr1* and *icr2* are distinct from any of the mutations or genes reported by Bartel and Fink (1995). We have not shown that either CW6-6 (*icr1*) or CW204 (*icr2*) is defective in IAA conjugate hydrolysis at the whole plant level. However, given the presence of several genes of which the products are capable of IAA conjugate hydrolysis, the loss of one hydrolytic enzyme might lead to changes in hydrolysis undetectable at the whole-plant level but that due to their tissue(s) of expression and action are nevertheless sufficient to modify the plant's sensitivity to growth inhibition by exogenous conjugate. Whether any of the genes defined by either our mutations or those of Bartel and Fink (1995) actually play a role in IAA conjugate metabolism and IAA homeostasis in vivo remains to be determined.

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